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Comparative Pharmacokinetics and Subacute Toxicity of Di(2-ethylhexyl) Phthalate (DEHP) in Rats and Marmosets: Extrapolation of Effects in Rodents to Man

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Certain phthalate esters and hypolipidemic agents are known to induce morphological and biochemical changes in the liver of rodents, which have been associated with an increased incidence of hepatocellular tumors in these species. There is evidence that hypolipidemic agents do not induce these effects in either subhuman primates or man. The oral and intraperitoneal administration of di(2-ethylhexyl) phthalate (DEHP) to the marmoset monkey at doses up to 5 mmole DEHP/kg body weight/day for 14 days did not induce morphological or biochemical changes in the liver or testis comparable with those obtained in rats given the same amount of DEHP. In the marmoset, the excretion profile of [^{14}C]-DEHP following oral, IP, and IV administration and the lower tissue levels of radioactivity demonstrated a considerably reduced absorption in this species compared to the rat.

The urinary metabolite pattern in the marmoset was in many respects qualitatively similar to but quantitatively different from that in the rat; the marmoset excreted principally conjugated metabolites derived from $\omega - 1$ oxidation. The pharmacokinetic differences between these two species indicate that the tissues of the marmoset are exposed to a level of DEHP metabolites equivalent to the complete absorption of a dose of Ca. 0.1 to 0.25 mmole DEHP/kg body weight/day without significant toxicological effects. These exposure levels are at least 100-fold greater than the worst estimates of incidental human exposure (ca. 0.0015 mmole/kg/day). They are comparable with the human therapeutic dose of many hypolipidemic drugs (ca. 0.15 mmole/kg/day), a dose at which it is claimed that there is an absence of morphological or biochemical changes to human or subhuman primate liver. The evidence suggests that in some nonrodent species the hepatocellular and testicular response to DEHP is considerably less than that in rodents and is dose-dependent.

Introduction

Di(2-ethylhexyl) phthalate (DEHP) and di(2-ethylhexyl) adipate (DEHA) are considered to be of low acute toxicity in a variety of animal species including man. However, repeat, oral administration of DEHP at high doses to rodent species produced biochemical and morphological changes in the liver (*1*) and testis (*2*). Recently DEHP was reported to induce liver tumors in F344 rats and B6C3F$_1$ mice and DEHA in B6C3F$_1$ mice in 2-year feeding experiment at maximally tolerated doses, whereas several other substances related to phthalic acid did not (*3*). Previous carcinogenicity studies on various phthalate esters had not shown similar effects at lower dose levels, but their validity has been questioned (*4*). Recent studies have confirmed an absence of covalent binding of DEHP and DEHA with DNA (*5*), which in conjunction with other negative short-term tests for mutagenicity gives additional support for the alternative hypothesis of reactive oxygen produced by the persistent proliferation of liver peroxisomes (*6*) as the initiator of the neoplastic transformation of liver cells. A critical factor in extrapolating from rodents to man is whether these effects occur in other species (*7*). Studies with the hypolipidemic drug ciprofibrate in several species showed peroxisome induction to be a dose-dependent rather than a species-specific phenomenon (*8*). A marked reduction

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in hepatic peroxisomal response has been observed in the Syrian hamster compared to the rat with DEHP (*9*), and similar reduced hepatic responses have been reported for the hypolipidemic agents clobuzarit (*10*) and clofibrate (*11*) in the subhuman primate species, the marmoset.

A preliminary report of a long-term chronic toxicity study of clofibrate in the marmoset at dose levels which produced hepatocellular tumors in the rat has, after 7 years, provided no evidence of hepatocellular carcinoma in this subhuman primate species (*12*).

A lack of peroxisome induction in human liver biopsies removed from patients receiving therapeutic doses of hypolipidemic agents has also been reported (*13*). Because of the absence or reduced biochemical and morphological response in primate livers following exposure to hypolipidemic drugs, we have studied the pharmacokinetics and subacute effects of DEHP in male and female marmosets.

Experimental

Materials

[^{14}C]-DEHP and [^{14}C]-MEHP, both uniformly labeled in the phenyl ring were synthesized by the Petrochemical and Plastic Division Imperial Chemical Industries Billingham, UK, to greater than 99% purity. DEHP was obtained from British Petroleum Chemicals, Hull Works, North Humberside, UK. The purity was determined by gas chromatography to be 99.7%. The mass spectrum was identical with that of a library specimen. All other reagents were Analar (analytical) grade where possible.

Animals

Adult male and female Wistar-derived albino rats (130–190 g body weight, between 6 and 8 weeks old) of the Alderley Park Specific Pathogen-Free strain (Alpk/Ap) were housed in suspended stainless steel wire mesh cages. The animals were fed throughout the studies with a standard rat PCD diet (BP Nutrition, Witham, Essex) and allowed tap water *ad libitum*. Adult male and female marmosets (*Callithrix jacchus*), 250–400 g body weight, 12–18 months old for the oral studies and 450 g, 24 months old, for the intraperitoneal study, were bred at Imperial Chemical Industries, Pharmaceuticals Division (Alderley Park, Cheshire, UK). Animals were fed a daily meal of Mazuri Primate diet, fruit malt bread, vitamin supplements of Vitetrin (E.R. Squibb and Sons Ltd.) and Bemax (Glaxo Ltd.) each given twice weekly in the diet. Water was allowed *ad libitum*.

Toxicity Studies

Groups of ten male and ten female rats were used for the studies with a period of 6 days acclimatization. Male and female rats were each given single oral doses of DEHP (2000 mg/kg body weight) in corn oil (10 mL/kg) daily for 14 consecutive days. Control animals received corn oil only (10 mL/kg). The animals were weighed each day prior to dosing in order to determine the daily dose level. Groups of five male and five female marmosets were used for the oral studies, and five male marmosets for the IP toxicity studies, with 7 days acclimatization prior to dosing. Each was given single oral gavage doses of DEHP (2000 mg/kg) daily for 14 consecutive days. Control animals received corn oil only (2 mL/kg). For the IP studies each animal received daily a single IP dose of 1000 mg/kg DEHP as a 50% (w/v) DEHP corn oil formulation for 14 consecutive days. Rats and marmosets were killed by inhalation of excess anesthetic. Immediately after death, blood was withdrawn from each animal by cardiac puncture; each animal was examined externally and by dissection for macroscopic abnormalities. Liver, kidneys, testes, and brain were weighed, and sections of selected tissues were taken for microscopic examination; samples of liver were also taken for biochemical analysis.

Pharmacokinetic Studies

Animals were housed in glass metabolism cages, equipped for the complete collection of urine and feces. Animals received approximately 20 to 25 μCi ^{14}C-DEHP/kg.

In the multiple dose studies ^{14}C-DEHP was administered orally daily for 14 days. Blood samples were taken at specific times on day 1 and day 14. Tissue samples were removed 24 hr after the last of 14 daily doses. Urine and feces were collected for 24-hr periods following administration on days 5 and 14.

In the single-dose studies, male marmosets received ^{14}C-DEHP by the IV (100 mg/kg), IP (1000 mg/kg) or oral (100 and 2000 mg/kg) routes. Urine and feces were collected at 24-hr intervals for up to 7 days. Residual tissue levels were determined at 7 days.

In separate studies, male marmosets were orally dosed with either 50 μCi ^{14}C-DEHP/kg or 50 μCi ^{14}C-mono-2-ethylhexyl phthalate (MEHP)/kg, both at 0.25 mmole/kg (equivalent to 100 mg DEHP or 70 mg MEHP/kg). Urine was collected at intervals and used for the qualitative and quantitative determination of the metabolite profile by GC and GC-MS.

Hepatic Enzyme Activity

Liver was homogenized in four volumes of 0.25 M sucrose/5 mM Tris-HCl (pH 7.4). The liver homogenate was then fractionated into a large particulate fraction (nuclei, mitochondria, peroxisomes, and lysosomes), a microsomal fraction, and cytosol. Several parameters were measured in the relevant subcellular preparations. Only the following are reported: catalase (*15*); microsomal cytochrome P-450 and cytochrome b_5 (*16*); microsomal ethoxycoumarin-*O*-deethylation (*17*) and lauric acid hydroxylation (*18*); CN-insensitive palmitoyl CoA oxidation (*19*) and α-glycerophosphate dehydrogenase (*20*).

Plasma Clinical Chemistry

Plasma triglyceride and cholesterol were measured as previously described (*14*).

Microscopy

Light Microscopy. Liver (left caudate and papillary lobes from the rat and right median and papillary lobes from the marmoset), kidney (left), pituitary, and testes were fixed in buffered formol saline, embedded in paraffin wax, and 5.5 μm sections stained with hematoxylin and eosin.

Electron Microscopy. Sections of the liver (right median lobe from the rat, right median and left lobes from the marmoset) were fixed in modified Karnovsky's mixed formaldehyde, gluteraldehyde fixative, and 1 mm slices postfixed in 1% buffered osmium tetroxide, dehydrated in graded acetones and embedded in Araldite resin (Ciba Geigy Ltd). Sections (1 μm) were stained with toluidine blue and used to select suitable areas for electron microscopy. Ultrathin sections (70–90 nm) stained with uranyl acetate and lead citrate were assessed visually, and micrographs recorded at ×24,900 magnification, for morphometric analysis of peroxisomes using a test grid of 320 points (*21*).

Preparation of Samples for Determination of Radioactivity

Urine. Urine samples were diluted to a standard volume (25 mL) with water, and duplicate aliquots were diluted to 1.0 mL with methanol prior to scintillation counting.

Feces, Tissues, and Blood. Fecal samples were freeze-dried overnight, and duplicate samples (approximately 50 mg) were combusted in a 306B Tri-Carb sample oxidizer (Packard Instruments Limited). Tissues, homogenized with an equal weight of water, and samples of blood (60–320 mg) absorbed on to cellulose pads, were combusted as above.

Measurement of Radioactivity

Radioactivity was determined by liquid scintillation counting of prepared samples by use of a Tri-Carb 460CD microprocessor-based liquid scintillation spectrometer (Packard Instruments Limited), automatically corrected for background and counting efficiencies (external standard quench correction curve data with ^{226}Ra as a gamma source). Samples were counted to a statistical precision of 1%.

Results and Discussion

Subacute Toxicity

Most of the published data on DEHP has been derived from studies with rodents. This study has compared the effects of the subacute administration of DEHP in a rodent species (rat) with those in a primate species (marmoset). The two best documented effects of DEHP in the rat are testicular atrophy and hepatomegaly. The present data demonstrate a reduction in body weight gain, testicular atrophy, and hepatomegaly in rats after 14 days oral administration at a high dose level of DEHP (2000 mg/kg/day) (Fig. 1). Although marmoset body weight was affected following administration of DEHP, changes in organ weight were not detected at high dose levels of DEHP (orally 2000 mg/kg/day or IP 1000 mg/kd/day). Induction of hepatomegaly following chronic DEHP administration has been previously studied in the dog (less affected than rat) and guinea pig (absence of effect) (*25*). While the rat, mouse, guinea pig, and ferret were susceptible to testicular atrophy following exposure to DEHP and the related plasticizer dibutyl phthalate (DBP), the hamster was resistant to their gonadal effects (*2*).

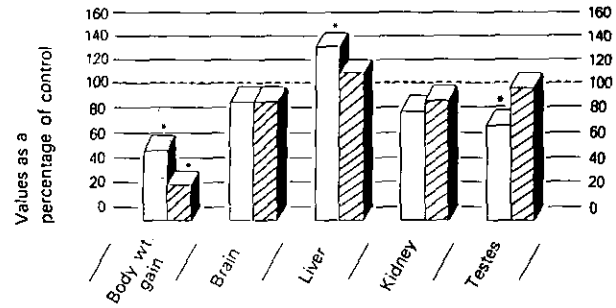

FIGURE 1. Body weights and organ weights: (□) rat; (▨) marmoset. The asterisk (*) denotes significantly different from control, $p < 0.05$.

Hepatic peroxisomes (Fig. 2) and peroxisomal enzymes (Fig. 3) were induced in both male and female rats, whereas the hypotriglyceridemic and hypocholesteremic effects (Fig. 4) were only observed in male but not female rats. The induction of peroxisomes and peroxisomal enzyme activity and the hypolipidemic effects were not detected following oral or IP administration of DEHP to the marmoset. The data from this present study indicate that the interrelationship of hepatomegaly, peroxisomal induction, and hypolipidemia is complex and appears to be dose- and species-dependent. Although, in the marmoset, there was an increase in catalase activity, there was not an increase in cyanide-insensitive acyl oxidase, the peroxisome marker enzyme. Hypolipidemic agents that induce peroxisomal activity in rats showed species selectivity for this effect and notably, no peroxisome proliferation was detected in the marmoset (*10*). However, other nonrodents, including primates, have been shown to be responsive to the peroxisome-inducing activity of the potent hypolipidemic drug ciprofibrate (*8*).

This study has indicated the induction of a rat hepatic cytochrome P-450 with high activity towards the C-11 and C-12 hydroxylation of lauric acid. A 10-fold increase in this activity was seen, compared to a 2- to 3-fold induction of ethoxycoumarin-*O*-deethylation. The induction

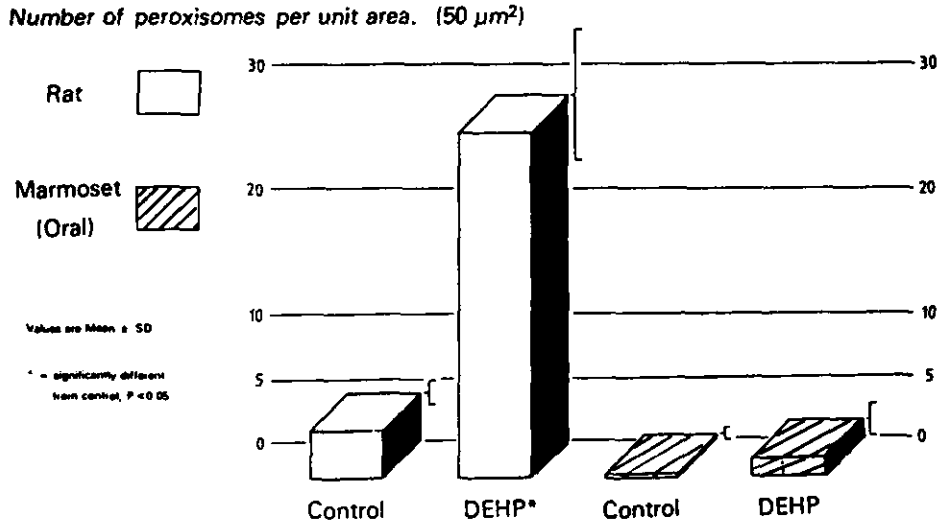

FIGURE 2. Quantitation of peroxisomes. Values are mean ± SD. The asterisk (*) denotes significantly different from control, $p < 0.05$.

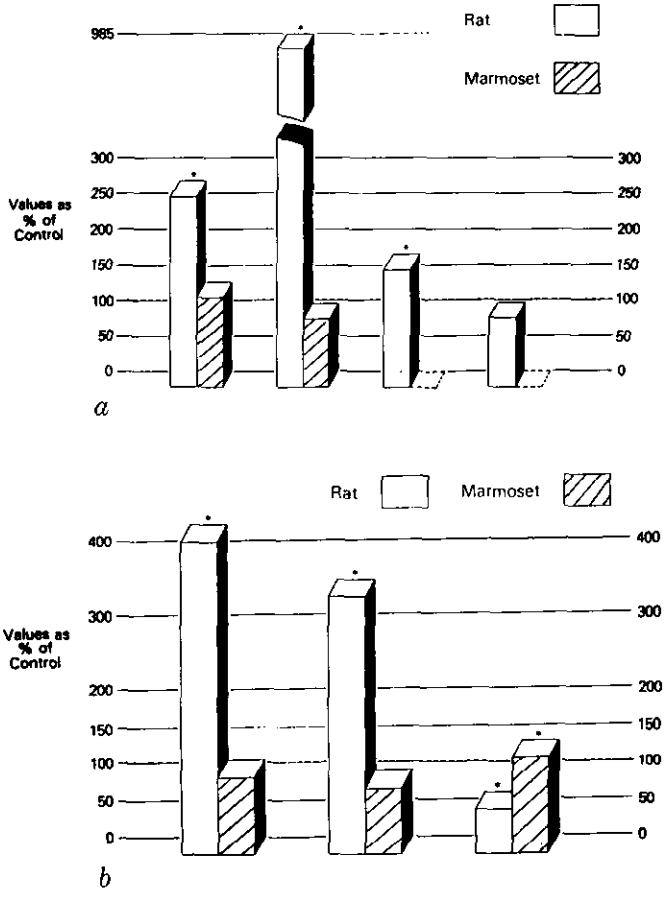

FIGURE 3. Liver biochemistry: (a) hepatic microsomal enzyme activities of (left to right) ethoxycoumarin-*O*-deethylation, lauric acid hydroxylation, cytochrome P-450, and cyctochrome B_5 in the rat and marmoset; (*b*) enzyme activities of 15,000*g* liver fraction to (left to right) CN-insensitive palmitoyl CoA oxidation, α-glycerophosphate dehydrogenase, and catalase. The asterisks (*) denote significantly different from control, $p < 0.05$.

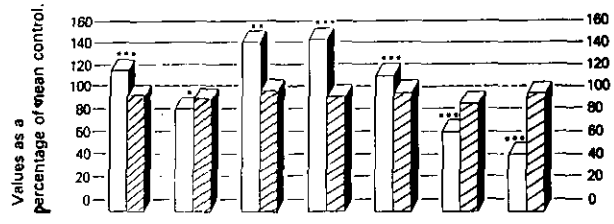

FIGURE 4. Clinical chemistry of (left to right) urea, glucose, ALP, A:T, albumin, cholesterol, and triglyceride in (□) the rat and (▨) the marmoset. Statistical significance relative to control: (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$.

of a microsomal protein of molecular weight 56,000 was observed following DEHP administration to rats. Similar observations have been reported following the treatment of rats with various peroxisomal proliferation hypolipidemic agents (*18*). While the significance of the induction of this hydroxylation is not yet known, one may speculate that it may have a prominent role in the biotransformation of the alkyl side chains of DEHP.

Mammalian peroxisomes contain a number of oxidases, one of prime importance being the cyanide-insensitive acyl CoA oxidation system which has been shown to be markedly elevated in rodents but not marmosets following exposure to DEHP. In contrast to the mitochondrial β-oxidation system, the peroxisomal system is not coupled to oxidative phosphorylation and generates H_2O_2 which is detoxified by the peroxisomal enzyme, catalase. Catalase is much less induced than the acyl CoA oxidases generating H_2O_2, so that an increased steady-state cellular concentration of H_2O_2 may occur. It is possible that elevated levels of H_2O_2 in hepatic cells may lead to genetic damage similar to that observed in cultured mammalian cells (*26*). Such changes in rodent hepatic cells following the subacute administration of high doses of DEHP may

be of particular importance to the increased incidence of hepatocellular carcinomas and adenomas in mice and rats. The absence of evidence for interaction of DEHP with rodent hepatic DNA (*5*) and the high dose required to induce tumors suggests that DEHP belongs to a class of indirect nongenotoxic (epigenetic) carcinogens. The mechanism of such carcinogenicity may be associated with species-specific perturbation of cellular biochemistry (*27*). Consequently, the absence of such effects in the marmoset may be indicative that, as with hypolipidemic drugs, the induction of peroxisomes by DEHP is species- and/or dose-dependent. Therefore, the hepatocellular tumors in rodents will not occur at dose levels that do not produce the necessary perturbation of cellular biochemistry, and a threshold dose for the tumorigenicity will exist.

Disposition of DEHP in the Marmoset

Subacute Administration. In both rat and marmoset, multiple oral administrations of ^{14}C-DEHP at 2000 mg/kg. body weight/day did not modify the proprotion of dose excreted in the urine or feces in either male or female animals. Compared with a single dose, the marmoset excreted 2% of the administered dose in the urine compared with about 50% excreted by the rat (Fig. 5). The levels of DEHP or its metabolites in the tissues of the marmoset 24 hr after the fourteenth and final dose of [^{14}C]-DEHP were between one-fifth and one-tenth of the levels in the rat at the corresponding time point (Fig. 6), confirming the reduced bioavailability of DEHP in the marmoset.

Absorption and Routes of Excretion Following a Single Dose of DEHP. The cumulative excretion in urine and feces following IV administration to marmosets shows approximately 40% of the dose excreted in urine and approximately 20% in the feces (Fig. 7). This indicates an approximate 2:1 split between the urinary and unreabsorbed biliary (fecal) routes of excretion in the marmoset. A much smaller proportion of the dose (14%) was excreted following IP administration, with the urinary and biliary (fecal) excretion in a similar 2:1 ratio. This suggests that following oral administration at the 100 mg/kg dose the 30% excretion in the urine probably reflects 45% absorption of the dose with a 15% excretion in feces via the biliary circulation. A large proportion of both parenterally administered DEHP doses (IV and IP) remained within the marmoset at 7 days. Following IV administration 28% of the dose remained in the lungs with minimal levels in other tissues, which probably reflects entrapment of the insoluble DEHP from the IV emulsion by the alveolar capillaries. Following IP administration, 85% of the dose remained as unabsorbed DEHP in the peritoneal cavity with minimal amounts in the tissues. The residual levels in the tissues at 7 days following oral administration of 2000 mg DEHP/kg were about one-fifth of the IV dose of 100 mg DEHP/kg (Fig. 8). Contamination of tissue by DEHP remaining in the peritoneal cavity prevents any interpretation of the tissue levels following IP administration. When the data are expressed as milligrams of DEHP equivalents excreted in urine (Table 1) at the larger oral dose, there is a reduction in the absorption of DEHP from the intestinal tract of the marmoset. The amount excreted in urine is more consistent to that expected for a 150 to 200 mg/kg dose. While the IP route provides an alternative route for parenteral administration, it also provides only a limited absorption of material equivalent to a 300 mg/kg dose. A comparison of the blood level profiles for the three routes of administration confirms the dose-dependent absorption of DEHP in the marmoset (Fig. 9). A 20-fold increase in the oral dose from 100 to 2000 mg/kg showed only a 2-fold increase in the amount absorbed.

There is a significant difference in absorption of large

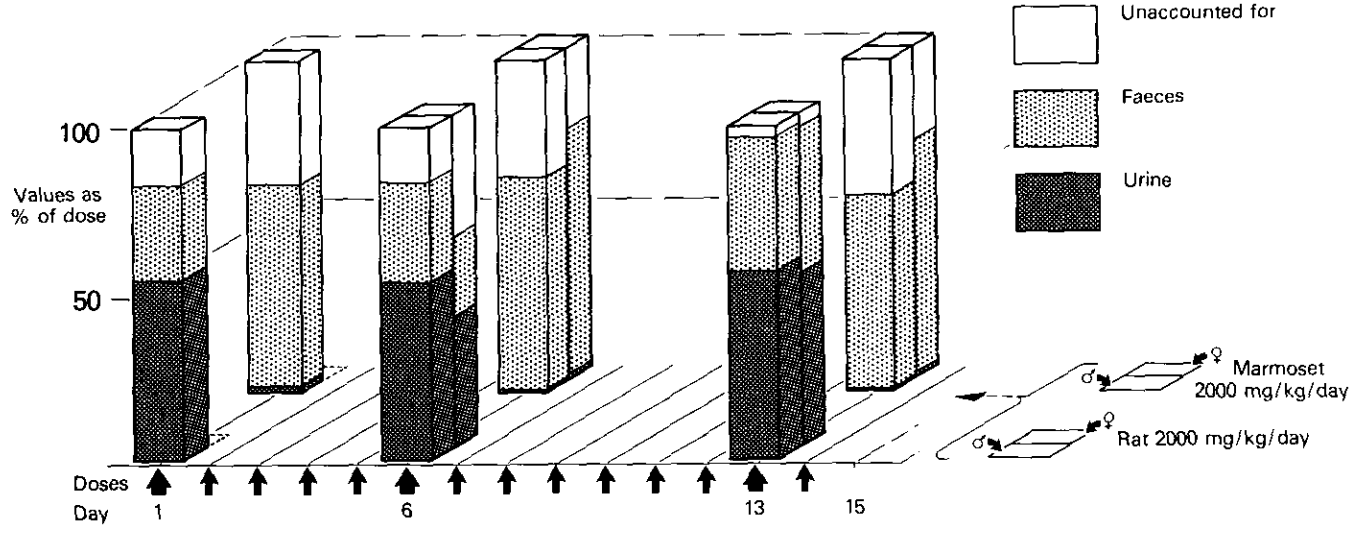

FIGURE 5. Excretion profile following multiple oral administration in the rat and marmoset.

doses of DEHP between rat and marmoset. At an oral dose of 2000 mg/kg, marmoset tissue is exposed to DEHP and its metabolties at an approximately equivalent level to that expected for rat tissues following an oral dose of 200 mg/kg to the rat. The IP route provides a slightly increased bioavailability to that of the oral route, but it also shows dose-dependent absorption. Because of the insolubility of DEHP, distribution of large IV doses is limited by the sequestering effect of lung tissue. The data suggest that DEHP is not as readily hydrolyzed by marmoset lipases; therefore, it is not as readily absorbed by this species. The activity of marmoset lipases appears to be much less than that of the rat (B. G. Lake, personal communication).

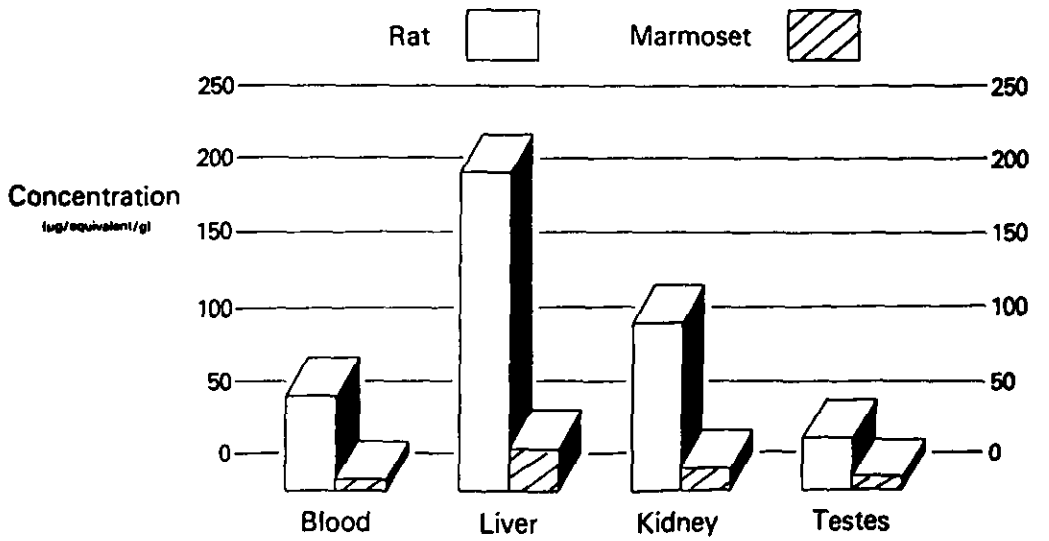

FIGURE 6. Blood and tissue levels following multiple oral administration of DEHP. Levels of radioactivity expressed as μg equivalents of DEHP 24 hr after the final dose of ^{14}C-DEHP.

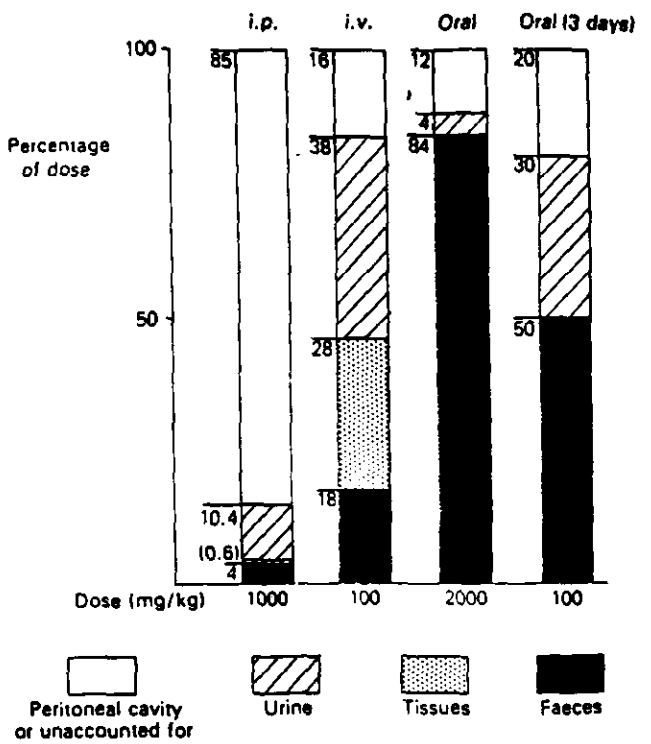

FIGURE 7. Proportion of dose in marmoset excreta and tissues 7 days after administration of ^{14}C-DEHP.

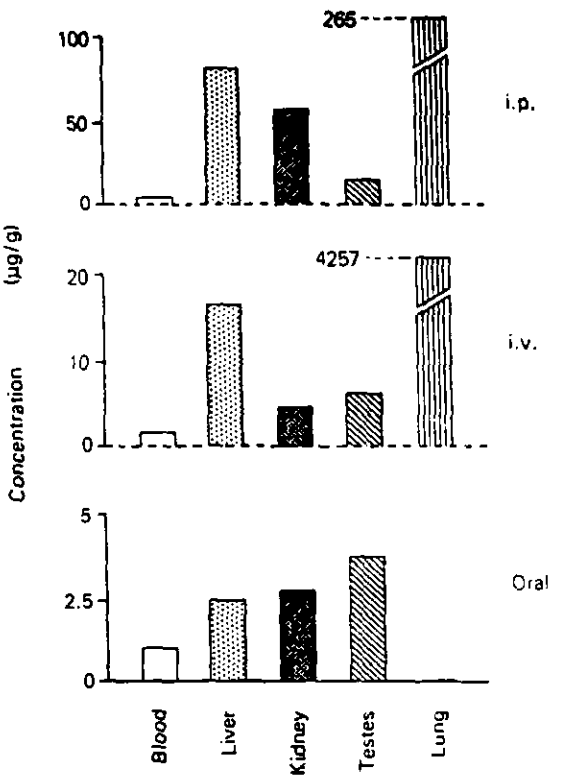

FIGURE 8. Tissue levels of DEHP and its metabolites 7 days after administration of ^{14}C-DEHP in the marmoset.

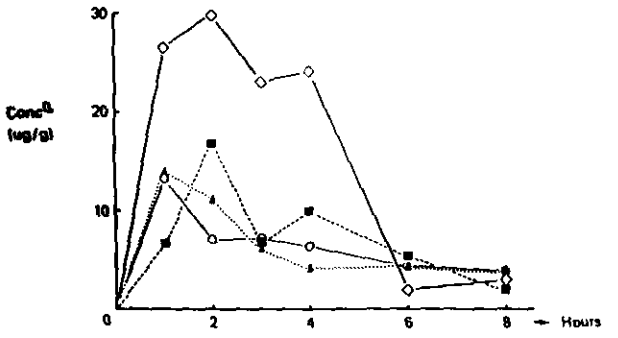

FIGURE 9. Marmoset blood levels of DEHP and its metabolites: (◇) oral, 200 mg/kg (42 mg excreted/24 hr), AUC_8 = 123; (■) oral, 100 mg/kg (20 mg excreted/24 hr), AUC_8 = 58; (▲) IP, 1000 mg/kg (20 mg excreted/24 hr), AUC_8 = 49; (○) IV, 100 mg/kg (14 mg excreted/24 hr), AUC_8 = 56.

Table 1. Amount of dose excreted after administration of ^{14}C-DEHP to male marmosets (N = 3).

Route	Dose, mg/kg	Amount excreted, mg		
		Urine	Feces	Total
IV	100	38	18	56
IP	1000	104	37	141
Oral	100	29	26	55
Oral	2000	74	1674	1758

Biotransformation of DEHP by the Marmoset

It has been established that DEHP is hydrolyzed by nonspecific pancreatic lipases to its monoester, MEHP, which is subsequently oxidized probably by enzymes of the ω, ω-1 and β-oxidation pathways to a variety of metabolites (*22,24*) (Fig. 10). Species differences in metabolism of DEHP have been reported (*28–30*).

In general there are only quantitative differences rather than qualitative differences in the metabolite profiles of the phase 1 oxidations between species. However, species other than the rat appear to excrete conjugated metabolites in the urine. In the marmoset, the urinary metabolite profiles following oral administration of a single dose of either [^{14}C]-DEHP or [^{14}C]-MEHP were similar (Table 2). The majority of the metabolites were excreted in mainly conjugated forms, probably glucuronides. This excretion of the conjugated metabolites is similar to other primate species, but dissimilar to the rat. In addition there were more ω-1 oxidation products excreted by the marmoset compared to the rat (Table 3).

From this study, the metabolism of DEHP by the marmoset is comparable to that of other primates (*30*) and shows the same characteristic differences from the rat as other primate species.

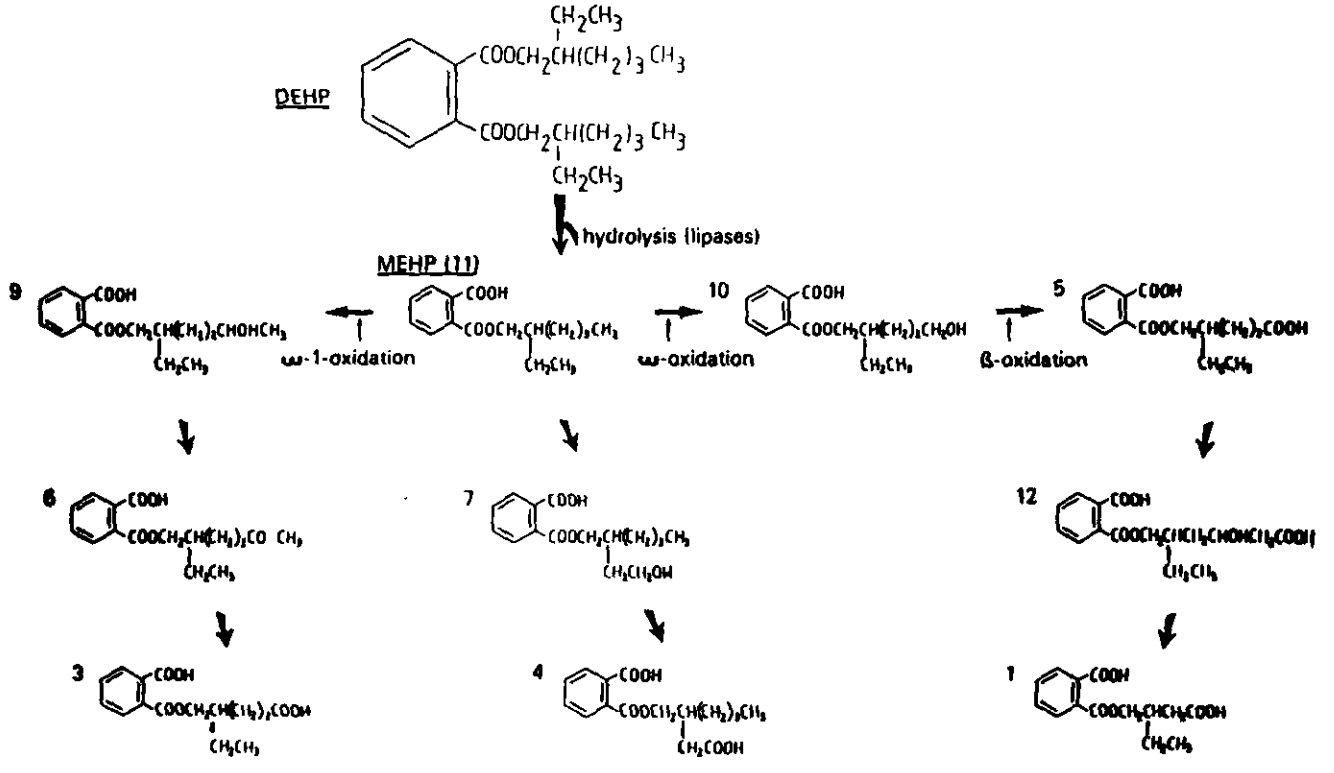

FIGURE 10. Metabolism of DEHP in the rat.

Table 2. *In vivo* metabolism of DEHP and MEHP in male marmosets following the oral administration of a single dose at 0.25 mmole/kg of either [^{14}C]-MEHP or [^{14}C]-DEHP.

		Percentage of metabolite in 0–8 hr urine fraction			
		DEHP		MEHP	
	Metabolite no[a].	Hydrolyzed	% Conjugated	Hydrolyzed	% Conjugated
MEHP	11	17 ± 3	30	19 ± 3	42
ω-oxidation					
Hexyl chain	1	2 ± 0.2	(36)	7 ± 10	42
	5	7 ± 1	63	6 ± 2	63
	10	1 ± 0.4	44	1 ± 0.4	47
	12	0	0	0	0
Ethyl chain	2	1 ± 0.2	0	1 ± 0.5	0
	4	3 ± 2	23	3 ± 2	33
	7	7 ± 3	66	6 ± 4	69
Total ω oxidation		22 ± 1	54	24 ± 3	55
ω-1 oxidation					
Hexyl chain	3	2 ± 1	0	2 ± 1	0
	6	9 ± 2	80	8 ± 2	80
	9	52 ± 2	77	47 ± 11	78
	8	1 ± 0.2	11	4 ± 5	14
Total ω-1 oxidation		64 ± 2	75	61 ± 11	76
Percentage of dose recovered in urine fraction		17 ± 4		24 ± 13	

[a] See Fig. 10.

Table 3. Metabolism of DEHP excreted in urine folowing single dose.

	Metabolite μmole/kg body weight[a]		
	Rat[a]	Marmoset[b]	Peroxisomal induction[c]
DEHP dose administered			
μmole/kg	125	250	Activity
mg/kg	(50)	(100)	*in vitro*
Metabolites			
MEHP	Zero	11	+
ω-Oxidation products	16	14	−
ω-1 Oxidation products	11	34	±
Excreted as conjugate(s)	Zero	33	
Total excreted	30	60	

[a] 24 hr.
[b] 8 hr.
[c] Rat hepatocytes; + = *induces*; − = *does not induce*.

Conclusion

The data presented in this paper have demonstrated that the subacute effects of DEHP, such as hepatic peroxisome proliferation, are less in the marmoset than in the rat when a large dose of DEHP (5 mmole DEHP/kg/day) is administered orally. The bioavailability of DEHP in the marmoset at high doses is limited. However, at the tissue levels obtained in the marmoset there was no marked biochemical or morphological change observed. At comparable tissue levels following oral administration of 200 mg/kg to the rat, there are reports of such changes (*31*). Although the marmoset metabolizes DEHP, it shows the same characteristic differences from the rat as do other primate species. The marmoset appears to be less sensitive to the effects of peroxisome proliferators such as DEHP and the hypolipidemic drugs. These metabolic differences may explain this species difference. This is of particular interest because peroxisome proliferation has not been observed in liver biopsies obtained from humans who had received prolonged clinical treatment, with hypolipidemics (ca. 0.15 mmole hypolipidemic/kg/day) (*32–34*). This dose level is known to produce peroxisome proliferation acutely in the rat (in the range 0.1 to 0.25 mmole hypolipidemic/kg/day) (*31*). If the marmoset reflects more accurately the response in man, then the low levels of DEHP to which man is incidentally exposed (ca. 0.0015 mmole DEHP/kg/day) should not be of toxicological significance with regard to hepatocellular carcinoma.

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